invitrogen™

pEF6/His A, B, and C

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User Manual

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Contents and Storage

Shipping and Storage

pEF6/His vectors are shipped on wet ice. Upon receipt, store vectors at –20ºC.

Contents

20 µg each of pEF6/His A, B, and C are supplied at 0.5 µg/µL in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 in a total volume of 40 µL.

20 µg of pEF6/His/*lac*Z is supplied at 0.5 µg/µL in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 in a total volume of 40 µL.

Introduction

Product Overview

Description of the System

pEF6/His A, B, and C are 5.8 kb vectors designed for high-level expression and purification of recombinant proteins in mammalian cell lines. Refer to page 15 for specific features of the vectors that allow for purification and detection of expressed proteins. High-level stable and transient expression can be carried out in most mammalian cells. The vectors contain the following elements:

- The human elongation factor 1 α (hEF-1α) subunit promoter provides high-level expression in a wide range of mammalian cells (Kim *et al.*, 1990; Mizushima and Nagata, 1990; Uetsuki *et al.*, 1989). See page 13 for more information.
- Three reading frames to facilitate in-frame cloning with an N-terminal tag encoding the Xpress™ epitope and a polyhistidine (6xHis) metal-binding tag.
- Blasticidin resistance gene (*bsd*) for selection of stable cell lines (Kimura *et al.*, 1994).
- Episomal replication in cell lines that are latently infected with SV40 or that express the SV40 large T antigen (e.g., COS7).

The control plasmid, pEF6/His/*lac*Z, is included for use as a positive control for transfection, expression, and detection in the cell line of choice.

Experimental Outline

Use the following outline to clone and express your gene of interest in pEF6/His.

- Consult the multiple cloning sites depicted on pages 3–5 to determine which vector (A, B, or C) should be used to clone your gene in-frame with the N-terminal Xpress™ epitope and polyhistidine tag.
- Ligate your insert into the appropriate vector and transform into *E. coli*. Select transformants with 50 to 100 µg/mL ampicillin (or 50 µg/mL blasticidin).
- Analyze your transformants for the presence of insert by restriction digestion.
- Select a transformant with the correct restriction pattern and use sequencing to confirm that your gene is cloned in-frame with the N-terminal peptide.
- Transfect your construct into the cell line of choice using your own method of transfection. Generate a stable cell line, if desired.
- Test expression of your recombinant gene by western blot analysis or other functional assay. For ordering information about an antibody against the Xpress™ epitope, see page 17.
- To purify your recombinant protein, you may use a metal-chelating resin such as ProBond™. ProBond™ resin is available separately (see page 17 for ordering information).

Methods

Cloning into pEF6/His A, B, and C

Before Starting

Diagrams are provided on pages 3–5 to help you ligate your gene of interest in-frame with the N-terminal peptide. General considerations for ligation and transformation are listed below.

General Molecular Biology Techniques

For help with DNA ligations, *E. coli* transformations, restriction enzyme analysis, purification of single-stranded DNA, DNA sequencing, and DNA biochemistry, see *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 1989) or *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994) (See **References**, page 21).

Transformation Method

You may use any method of your choice for transformation. Chemical transformation is the most convenient for most researchers. Electroporation is the most efficient and the method of choice for large plasmids.

Maintaining pEF6/His

To propagate and maintain pEF6/His A,B, and C, use the supplied 0.5 µg/µl stock solution in TE, pH 8.0 to transform a *rec*A (recombination deficient), *end*A (endonuclease A deficient) *E. coli* strain, such as TOP10F′, INVαF´, or equivalent (see page 17 for ordering information). Select transformants on LB plates containing 50–100 µg/mL ampicillin or 50 µg/mL blasticidin.

Be sure to prepare a glycerol stock of each plasmid for long-term storage (see page 6).

Note

The pEF6/His vectors are fusion vectors. To ensure proper expression of your recombinant protein, you must clone your gene in-frame with the ATG at base pairs 1716–1718. This will create a fusion with the N-terminal polyhistidine tag, Xpress™ epitope, and the enterokinase cleavage site. The vector is supplied in three reading frames to facilitate cloning. See the diagrams on pages 3–5 to develop a cloning strategy.

If you wish to clone as close as possible to the enterokinase cleavage site, follow the guidelines below:

- Digest pEF6/His A, B, or C with *Kpn* I.
- Create blunt ends with T4 DNA polymerase and dNTPs. See Ausubel, *et al.*, 1994 for a detailed protocol.
- Clone your blunt-ended insert in-frame with the lysine codon (AAG) of the enterokinase recognition sequence.

Following enterokinase cleavage, no vector-encoded amino acid residues will be present in your protein.

Continued on next page

Cloning into pEF6/His A, B, and C, Continued

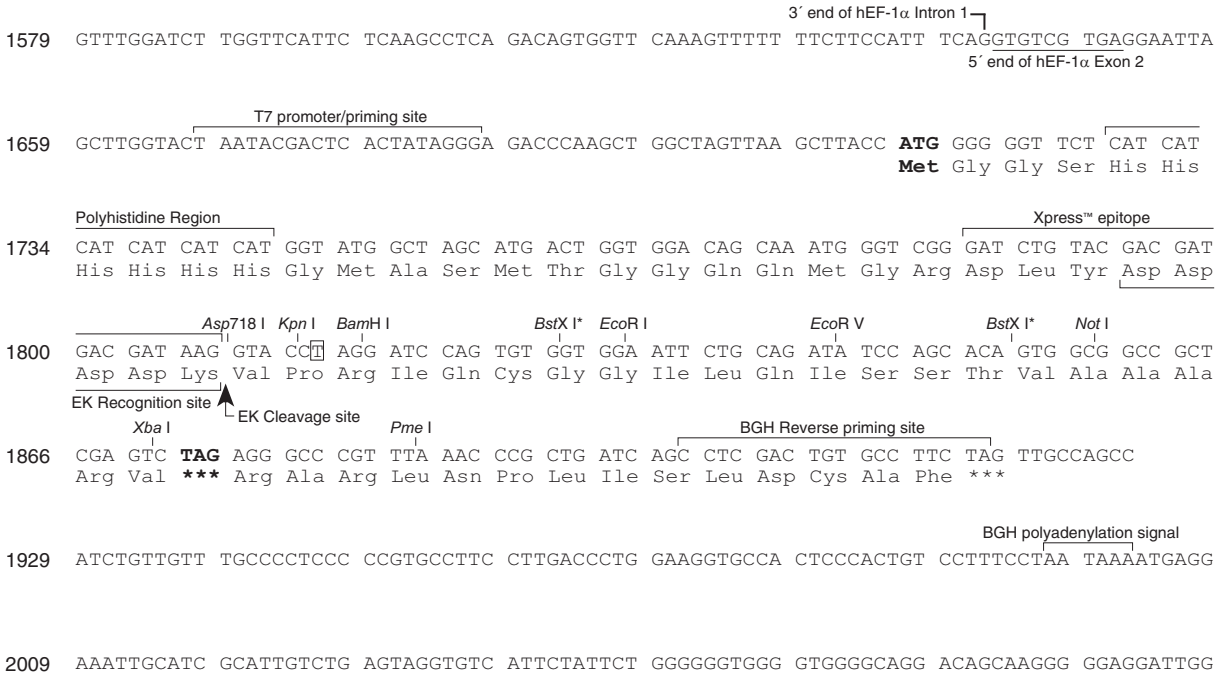
Kozak Sequence for Mammalian Expression

If you will be recombining your entry clone with a destination vector for mammalian expression, your insert should contain a Kozak consensus sequence with an ATG initiation codon for proper initiation of translation (Kozak, 1987; Kozak, 1990; Kozak, 1991). An example of a Kozak consensus sequence is provided below. Other sequences are possible, but the G or A at position –3 and the G at position +4 (shown in bold) illustrates the most commonly occurring sequence with strong consensus. Replacing one of the two bases at these positions provides moderate consensus, while having neither results in weak consensus. The ATG initiation codon is shown underlined.

(G/A)NNATG**G**

Multiple Cloning Site of pEF6/His A

Below is the multiple cloning site for pEF6/His A. Restriction sites are labeled to indicate the cleavage site. The boxed nucleotide indicates the variable region. **Note that there is a stop codon after the *Xba* I site.** The multiple cloning site has been confirmed by sequencing and functional testing. **The sequence may be downloaded from www.invitrogen.com** or requested from Technical Support (see page 18). For more information on the hEF-1α promoter, see page 13.


3´ end of hEF-1α Intron 1
1579 GTTTGGATCT TGGTTCATTC TCAAGCCTCA GACAGTGGTT CAAAGTTTTT TTCTTCCATT TCAGGTGTCG TGAGGAATTA
5´ end of hEF-1α Exon 2
T7 promoter/priming site
1659 GCTTGGTACT AATACGACTC ACTATAGGGA GACCCAAGCT GGCTAGTTAA GCTTACC ATG GGG GGT TCT CAT CAT
Met Gly Gly Ser His His
Polyhistidine Region
Xpress™ epitope
1734 CAT CAT CAT CAT GGT ATG GCT AGC ATG ACT GGT GGA CAG CAA ATG GGT CGG GAT CTG TAC GAC GAT
His His His His Gly Met Ala Ser Met Thr Gly Gly Gln Gln Met Gly Arg Asp Leu Tyr Asp Asp
Asp718 I Kpn I BamH I BstX I* EcoR I EcoR V BstX I* Not I
1800 GAC GAT AAG GTA CCT AGG ATC CAG TGT GGT GGA ATT CTG CAG ATA TCC AGC ACA GTG GCG GCC GCT
Asp Asp Lys Val Pro Arg Ile Gln Cys Gly Gly Ile Leu Gln Ile Ser Ser Thr Val Ala Ala Ala
EK Recognition site
EK Cleavage site
Xba I
Pme I
BGH Reverse priming site
1866 CGA GTC TAG AGG GCC CGT TTA AAC CCG CTG ATC AGC CTC GAC TGT GCC TTC TAG TTGCCAGCC
Arg Val *** Arg Ala Arg Leu Asn Pro Leu Ile Ser Leu Asp Cys Ala Phe ***
BGH polyadenylation signal
1929 ATCTGTTGTT TGCCCCTCCC CCGTGCCTTC CTTGACCCTG GAAGGTGCCA CTCCCACTGT CCTTTCCTAA TAAAATGAGG
2009 AAATTGCATC GCATTGTCTG AGTAGGTGTC ATTCTATTCT GGGGGGTGGG GTGGGGCAGG ACAGCAAGGG GGAGGATTGG

*There are two *Bst*X I sites in the multiple cloning site.

Continued on next page

Cloning into pEF6/His A, B, and C, Continued

Multiple Cloning Site of pEF6/His B

Below is the multiple cloning site for pEF6/His B. Restriction sites are labeled to indicate the cleavage site. The boxed nucleotides indicate the variable region. The multiple cloning site has been confirmed by sequencing and functional testing. **The sequence may be downloaded from www.invitrogen.com** or requested from Technical Support (see page 18). For more information on the hEF-1α promoter, see page 13.

```
                                                                  3´ end of hEF-1α Intron 1
1579  GTTTGGATCT TGGTTCATTC TCAAGCCTCA GACAGTGGTT CAAAGTTTTT TTCTTCCATT TCAGGTGTCG TGAGGAATTA
                                                                                5´ end of hEF-1α Exon 2

                          T7 promoter/priming site
1659  GCTTGGTACT AATACGACTC ACTATAGGGA GACCCAAGCT GGCTAGTTAA GCTTACC ATG GGG GGT TCT CAT CAT
                                                                     Met Gly Gly Ser His His

      Polyhistidine Region                                                    Xpress™ epitope
1734  CAT CAT CAT CAT GGT ATG GCT AGC ATG ACT GGT GGA CAG CAA ATG GGT CGG GAT CTG TAC GAC GAT
      His His His His Gly Met Ala Ser Met Thr Gly Gly Gln Gln Met Gly Arg Asp Leu Tyr Asp Asp

                  Asp718 I  Kpn I Bsu36 I  BamH I          BstX I*   EcoR I              EcoR V          BstX I*    Not I
1800  GAC GAT AAG GTA CCT AAG GAT CCA GTG TGG TGG AAT TCT GCA GAT ATC CAG CAC AGT GGC GGC CGC
      Asp Asp Lys Val Pro Lys Asp Pro Val Trp Trp Asn Ser Ala Asp Ile Gln His Ser Gly Gly Arg
      EK Recognition site  EK Cleavage site
            Xba I                    Pme I                                BGH Reverse priming site
1866  TCG AGT CTA GAG GGC CCG TTT AAA CCC GCT GAT CAG CCT CGA CTG TGC CTT CTA GTT GCC AGC CAT
      Ser Ser Leu Glu Gly Pro Phe Lys Pro Ala Asp Gln Pro Arg Leu Cys Leu Leu Val Ala Ser His

1932  CTG TTG TTT GCC CCT CCC CCG TGC CTT CCT TGA CCCT GGAAGGTGCC ACTCCCACTG TCCTTTCCTA
      Leu Leu Phe Ala Pro Pro Pro Cys Leu Pro ***

      BGH polyadenylation signal
1999  ATAAAATGAG GAAATTGCAT CGCATTGTCT GAGTAGGTGT CATTCTATTC TGGGGGGTGG GGTGGGGCAG GACAGCAAGG

2079  GGGAGGATTG GGAAGACAAT AGCAGGCATG CTGGGGATGC GGTGGGCTCT ATGGCTTCTG AGGCGGAAAG AACCAGCTGG
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*There are two *Bst*X I sites in the multiple cloning site.

Continued on next page

Cloning into pEF6/His A, B, and C, Continued

Multiple Cloning Site of pEF6/His C

Below is the multiple cloning site for pEF6/His C. Restriction sites are labeled to indicate the cleavage site. **Note that there is a stop codon within the *Pme* I site.** The multiple cloning site has been confirmed by sequencing and functional testing. **The sequence may be downloaded from www.invitrogen.com** or requested from Technical Support (see page 18). For more information on the hEF-1α promoter, see page 13.

3´ end of hEF-1α Intron 1
1579 GTTTGGATCT TGGTTCATTC TCAAGCCTCA GACAGTGGTT CAAAGTTTTT TTCTTCCATT TCAGGTGTCG TGAGGAATTA
5´ end of hEF-1α Exon 2
T7 promoter/priming site
1659 GCTTGGTACT AATACGACTC ACTATAGGGA GACCCAAGCT GGCTAGTTAA GCTTACC ATG GGG GGT TCT CAT CAT
Met Gly Gly Ser His His
Polyhistidine Region
Xpress™ epitope
1734 CAT CAT CAT CAT GGT ATG GCT AGC ATG ACT GGT GGA CAG CAA ATG GGT CGG GAT CTG TAC GAC GAT
His His His His Gly Met Ala Ser Met Thr Gly Gly Gln Gln Met Gly Arg Asp Leu Tyr Asp Asp
Asp718 I Kpn I BamH I
BstX I* EcoR I
EcoR V
BstX I* Not I
1800 GAC GAT AAG GTA CCA GGA TCC AGT GTG GTG GAA TTC TGC AGA TAT CCA GCA CAG TGG CGG CCG CTC
Asp Asp Lys Val Pro Gly Ser Ser Val Val Glu Phe Cys Arg Tyr Pro Ala Gln Trp Arg Pro Leu
EK Recognition site
EK Cleavage site
Xba I
Pme I
BGH Reverse priming site
1866 GAG TCT AGA GGG CCC GTT TAA AC CCGCTGATCA GCCTCGACTG TGCCTTCTAG TTGCCAGCCA TCTGTTGTTT
Glu Ser Arg Gly Pro Val ***
BGH polyadenylation signal
1939 GCCCCTCCCC CGTGCCTTCC TTGACCCTGG AAGGTGCCAC TCCCACTGTC CTTTCCTAAT AAAATGAGGA AATTGCATCG
2019 CATTGTCTGA GTAGGTGTCA TTCTATTCTG GGGGGTGGGG TGGGGCAGGA CAGCAAGGGG GAGGATTGGG AAGACAATAG

*There are two *Bst*X I sites in the multiple cloning site.

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E. coli Transformation

Transform the ligation mixtures into a competent *rec*A, *end*A *E. coli* strain (e.g., TOP10F´, INVαF´) and select on LB plates containing 50–100 µg/mL ampicillin or 50 µg/mL blasticidin. Select 10–20 clones and analyze for the presence and orientation of your insert.

Sequence your construct with the T7 Forward and BGH Reverse primers (see page 17 for ordering information) to confirm that your gene is fused in-frame with the Xpress™ epitope and the N-terminal polyhistidine tag. Refer to the diagrams on pages 3–5 for the sequences and locations of the priming sites. For your convenience, Invitrogen offers a custom primer service. For more information, visit www.invitrogen.com or call Technical Support (see page 18).

Primer	Sequence
T7 Forward	5′-TAATACGACTCACTATAGGG-3′
BGH Reverse	5′-TAGAAGGCACAGTCGAGG-3′

Preparing a Glycerol Stock

After identifying the correct clone, purify the colony and make a glycerol stock for long-term storage. It is also a good idea to keep a DNA stock of your plasmid at –20°C.

1. Streak the original colony on an LB plate containing 50 µg/mL ampicillin or 50 µg/mL blasticidin. Incubate the plate at 37°C overnight.
2. Isolate a single colony and inoculate into 1–2 mL of LB containing 50 µg/mL ampicillin (or 50 µg/mL blasticidin).
3. Grow the culture to mid-log phase (OD_{600} = 0.5–0.7).
4. Mix 0.85 mL of culture with 0.15 mL of sterile glycerol and transfer to a cryovial.
5. Store at –80°C.

Applying Selective Pressure

Take some (if not all) of the following precautions to prevent your clone from being "overrun" by background contaminants:

- **Use carbenicillin instead of ampicillin.** Carbenicillin is more stable than ampicillin, and allows for a longer period of selective pressure.
- **Increase the antibiotic concentration.** More antibiotic means that your clones will not be overwhelmed by β-lactamase buildup.
- **Periodically refresh plate media.** If you suspect that tubes/plates may be beginning to fail, spin them down, remove the old media, and replenish the wells with fresh LB media plus glycerol and antibiotic.

Streak clones on selective (preferably carbenicillin) LB agar plates. After about 12 hours, isolate colonies for downstream usage. This will isolate your desired clones from potential background contaminants.

Transfection

Introduction

After confirming that your construct is in the correct orientation and fused in-frame with the N-terminal peptide, transfect your cell line of choice. Include the positive control vector and a mock transfection to evaluate your results.

Plasmid Preparation

Plasmid DNA for transfection into eukaryotic cells must be clean and free from phenol and sodium chloride. Contaminants will kill the cells and salt will interfere with lipid complexing, decreasing transfection efficiency. Isolate DNA using the PureLink™ HiPure Miniprep Kit, the PureLink™ HiPure Midiprep Kit (see page 17 for ordering information), or CsCl gradient centrifugation.

Methods of Transfection

For established cell lines (e.g., HeLa), consult original references or the supplier of your cell line for the optimal method of transfection. Precisely follow the protocol for your cell line. Pay particular attention to medium requirements, when to pass the cells, and at what dilution to split the cells. Further information is provided in *Current Protocols in Molecular Biology* (**Reference** section, page 21).

Methods for transfection include calcium phosphate (Chen and Okayama, 1987; Wigler *et al.*, 1977), lipid-mediated (Felgner *et al.*, 1989; Felgner and Ringold, 1989) and electroporation (Chu *et al.*, 1987; Shigekawa and Dower, 1988). For high efficiency transfection in a broad range of mammalian cells, use Lipofectamine™ 2000 Reagent available from Invitrogen (see page 17).

Positive Control

pEF6/His/*lac*Z is provided as a positive control vector for mammalian transfection and expression (see page 16). It may be used to optimize transfection conditions for your cell line. The gene encoding β-galactosidase is expressed in mammalian cells under the control of the hEF-1α promoter. A successful transfection will result in β-galactosidase expression that can be easily assayed.

Assay for β-galactosidase Activity

You may assay for β-galactosidase expression by activity assay using cell-free lysates (Miller, 1972) or by staining the cells for activity. Invitrogen offers the β-Gal Assay Kit and the β-Gal Staining Kit (see page 17 for ordering information) for fast and easy detection of β-galactosidase expression.

Note

The N-terminal peptide containing the Xpress epitope and the polyhistidine tag will add approximately 3.4 kDa to your protein.

Purification

You will need 5×10^6 to 1×10^7 of **transfected** cells for purification of your protein on a 2 mL ProBond™ column (or other metal-chelating column). Refer to the manufacturer's instructions before attempting to purify your fusion protein. To prepare cells for lysis, see the protocol on page 12.

Continued on next page

Detecting Fusion Proteins

The Anti-Xpress™ Antibody is available from Invitrogen and can be used to detect expression of your fusion protein from pEF6/His (see page 17).

To detect the fusion protein by western blot, prepare a cell lysate from transfected cells. We recommend that you perform a time course to optimize expression of the fusion protein (*e.g.*, 24, 48, 72 hours, etc. after transfection). To lyse cells:

1. Wash cell monolayers (~10^6 cells) once with phosphate-buffered saline (PBS).
2. Scrape cells into 1 mL PBS and pellet the cells at 1,500 × *g* for 5 minutes.
3. Resuspend in 50 µL Cell Lysis Buffer (see below) or other suitable lysis buffer.
4. Incubate cell suspension at 37°C for 10 minutes to lyse the cells.
5. Centrifuge at 10,000 × *g* for 10 minutes to pellet nuclei and transfer the post-nuclear lysate to a new tube. Assay the lysate for protein concentration.

   Note: Do not use protein assays utilizing Coomassie Blue or other dyes. NP-40 interferes with the binding of the dye with the protein.
6. Add SDS-PAGE sample buffer to a final concentration of 1X and boil the sample for 5 minutes.
7. Load 20 µg of lysate onto an SDS-PAGE gel and electrophorese. Use the appropriate percentage of acrylamide to resolve your fusion protein.

Cell Lysis Buffer

50 mM Tris, pH 7.8
150 mM NaCl
1% Nonidet P-40

1. Prepare the solution from the following common stock solutions.

   For 100 mL, combine:

Stock Solution	Volume
1 M Tris base	5 mL
5 M NaCl	3 mL
Nonidet P-40	1 mL

2. Bring the volume to 90 mL with deionized water and adjust the pH to 7.8 with HCl.
3. Bring the volume to 100 mL. Store at room temperature.

Note: Protease inhibitors may be added at the following concentrations:

1 mM PMSF

1 µg/mL Pepstatin

1 µg/mL Leupeptin

Creating Stable Cell Lines

Introduction

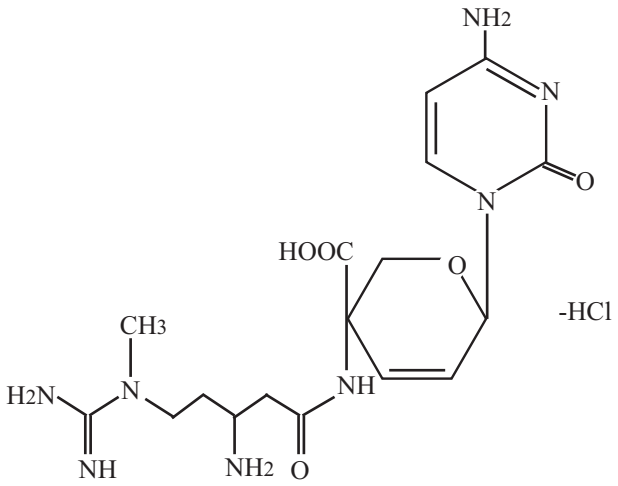
The pEF6/His vectors contain the blasticidin resistance gene for selection of stable cell lines using blasticidin. Test the sensitivity of your mammalian host cell to blasticidin as natural resistance varies among cell lines. General information and guidelines are provided below for your convenience.

Blasticidin

Blasticidin S HCl is a nucleoside antibiotic isolated from *Streptomyces griseochromogenes* which inhibits protein synthesis in both prokaryotic and eukaryotic cells (Takeuchi *et al.*, 1958; Yamaguchi *et al.*, 1965). Resistance is conferred by expression of either one of two blasticidin S deaminase genes: *bsd* from *Aspergillus terreus* (Kimura *et al.*, 1994) or *bsr* from *Bacillus cereus* (Izumi *et al.*, 1991). These deaminases convert blasticidin S to a non-toxic deaminohydroxy derivative (Izumi *et al.*, 1991) .

Molecular Weight, Formula, and Structure

The formula for blasticidin is $C_{17}H_{26}N_8O_5$-HCl, and the molecular weight is 458.9. The diagram below shows the structure of blasticidin.



Handling Blasticidin

Always wear gloves, mask, goggles, and protective clothing (e.g., a laboratory coat) when handling blasticidin. Weigh out blasticidin and prepare solutions in a hood.

Continued on next page

Preparing and Storing Stock Solutions

Blasticidin may be obtained from Invitrogen in 50 mg aliquots (see page 17 for ordering information). Blasticidin is soluble in water and is generally used to prepare stock solutions of 5 to 10 mg/mL.

- Dissolve blasticidin in sterile water and filter-sterilize the solution.
- Aliquot in small volumes suitable for one time use (see last point below) and freeze at –20°C for long-term storage or store at 4°C for short term storage.
- Aqueous stock solutions are stable for 1–2 weeks at 4°C and 6–8 weeks at –20°C.
- The pH of the aqueous solution should not exceed 7 to prevent inactivation of blasticidin.
- Do not subject stock solutions to freeze/thaw cycles (**do not store in a frost-free freezer**).
- Upon thawing, use what you need and discard the unused portion.

Possible Sites for Linearization

To obtain stable transfectants, you may choose to linearize your vector before transfection. While linearizing your vector may not improve the efficiency of transfection, it increases the likelihood that the vector will not integrate in a way that disrupts the gene of interest. The table below lists unique sites that may be used to linearize your construct prior to transformation. **Other restriction sites are possible. Note that the cleavage site is indicated for versions A, B, and C of pEF6/His.** Be sure that your insert does not contain the restriction enzyme site you wish to use to linearize your vector.

Enzyme	Restriction Site (bp) (A, B, C)	Location	Supplier
Ssp I	4	Upstream of hEF-1α promoter	Invitrogen*
Aat II	122	Upstream of hEF-1α promoter	Many
Mlu I	351	Upstream of hEF-1α promoter	Invitrogen*
*Bst*1107 I	3751 (A), 3752 (B), 3750 (C)	End of SV40 poly A	Roche
Sap I	4014 (A), 4015 (B), 4013 (C)	Backbone	New England Biolabs
*Eam*1105 I	5023 (A), 5024 (B), 5022 (C)	Ampicillin gene	Roche
Fsp I	5245 (A), 5246 (B), 5244 (C)	Ampicillin gene	New England Biolabs
Sca I	5503 (A), 5504 (B), 5502 (C)	Ampicillin gene	Invitrogen*

*see page 17 for ordering information

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Creating Stable Cell Lines, Continued

Selection in Mammalian Cell Lines

To generate a stable cell line expressing your protein, you need to determine the minimum concentration of blasticidin required to kill your untransfected host cell line. Typically, concentrations between 2 and 10 µg/mL blasticidin are sufficient to kill the untransfected host cell line. Test a range of concentrations (see below) to determine the minimum concentration necessary for your cell line.

1. Seed cells (2×10^5 cells/60 mm plate) for each time point and allow cells to adhere overnight.
2. The next day, substitute culture medium with medium containing varying concentrations of blasticidin (e.g. 0, 1, 3, 5, 7.5, and 10 µg/mL).
3. Replenish the selective medium every 3–4 days. Cells sensitive to blasticidin will round up and detach from the plate. Dead cells will accumulate in the medium.
4. Count the number of viable cells at regular intervals to determine the appropriate concentration of blasticidin that prevents growth.

Selecting Stable Integrants

Once the appropriate blasticidin concentration is determined, generate a stable cell line with your construct. Colonies can generally be identified in 7 to 10 days with complete selection and expansion in 2 weeks.

1. Transfect your cells using the appropriate protocol for your cell line. Include a sample of untransfected cells as a negative control.
2. After transfection, wash the cells once with 1X PBS and add fresh medium to the cells.
3. 48 hours after transfection, split the cells into fresh medium containing blasticidin at the appropriate concentration for your cell line. Split the cells such that they are no more than 25% confluent.
4. Replenish selective medium every 3–4 days until blasticidin-resistant colonies are detected.
5. Pick and expand colonies.

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Preparing Cells for Lysis

Use the procedure below to prepare cells for lysis prior to purifying your protein on ProBond™. You will need 5×10^6 to 1×10^7 cells for purification of your protein on a 2 mL ProBond™ column (see the ProBond™ Protein Purification manual).

1. Seed cells in five T-75 flasks or 2 to 3 T-175 flasks.
2. Grow the cells in selective medium until they are 80–90% confluent.
3. Harvest the cells by treating with trypsin-EDTA for 2 to 5 minutes or by scraping the cells in PBS.
4. Inactivate the trypsin by diluting with fresh medium (if necessary) and transfer the cells to a sterile microcentrifuge tube.
5. Centrifuge the cells at 250 × *g* for 5 minutes. Resuspend the cell pellet in PBS.
6. Centrifuge the cells at 250 × *g* for 5 minutes. You may lyse the cells immediately or freeze in liquid nitrogen and store at −70°C until needed.

Lysing Cells

If you are using ProBond™ resin, refer to the ProBond™ Protein Purification manual for details about sample preparation for chromatography.

If you are using another metal-chelating resin, refer to the manufacturer's instructions for recommendations on sample preparation.

Appendix

Human EF-1α Promoter

Description

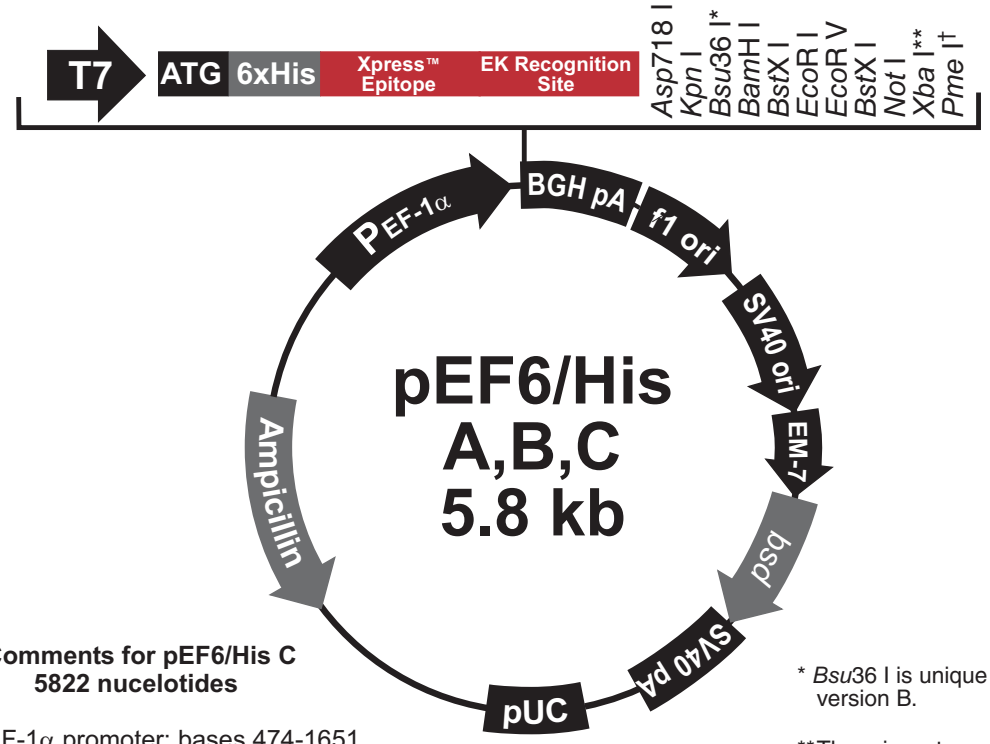
The diagram below shows the features of the hEF-1α promoter used in the pEF6/His vectors (Mizushima and Nagata, 1990). Features are marked as per Uetsuki, *et al.*, 1989.

5′ end of human EF-1α promoter
459 AAGGAGTGGG AATTGGCTCC GGTGCCCGTC AGTGGGCAGA GCGCACATCG CCCACAGTCC
519 CCGAGAAGTT GGGGGGAGGG GTCGGCAATT GAACCGGTGC CTAGAGAAGG TGGCGCGGGG
579 TAAACTGGGA AAGTGATGTC GTGTACTGGC TCCGCCTTTT TCCCGAGGGT GGGGGAGAAC
TATA box
Start of Transcription
639 CGTATATAAG TGCAGTAGTC GCCGTGAACG TTCTTTTTCG CAACGGGTTT GCCGCCAGAA
Exon I
5′ end of Intron 1
699 CACAGGTAAG TGCCGTGTGT GGTTCCCGCG GGCCTGGCCT CTTTACGGGT TATGGCCCTT
759 GCGTGCCTTG AATTACTTCC ACCTGGCTGC AGTACGTGAT TCTTGATCCC GAGCTTCGGG
819 TTGGAAGTGG GTGGGAGAGT TCGAGGCCTT GCGCTTAAGG AGCCCCTTCG CCTCGTGCTT
879 GAGTTGAGGC CTGGCCTGGG CGCTGGGGCC GCCGCGTGCG AATCTGGTGG CACCTTCGCG
939 CCTGTCTCGC TGCTTTCGAT AAGTCTCTAG CCATTTAAAA TTTTTGATGA CCTGCTGCGA
999 CGCTTTTTTT CTGGCAAGAT AGTCTTGTAA ATGCGGGCCA AGATCTGCAC ACTGGTATTT
Sp 1
1059 CGGTTTTTGG GGCCGCGGGC GGCGACGGGG CCCGTGCGTC CCAGCGCACA TGTTCGGCGA
Sp 1
1119 GGCGGGGCCT GCGAGCGCGG CCACCGAGAA TCGGACGGGG GTAGTCTCAA GCTGGCCGGC
Sp 1
Sp 1
1179 CTGCTCTGGT GCCTGGCCTC GCGCCGCCGT GTATCGCCCC GCCCTGGGCG GCAAGGCTGG
1239 CCCGGTCGGC ACCAGTTGCG TGAGCGGAAA GATGGCCGCT TCCCGGCCCT GCTGCAGGGA
Sp 1
1299 GCTCAAAATG GAGGACGCGG CGCTCGGGAG AGCGGGCGGG TGAGTCACCC ACACAAAGGA
Ap 1
1359 AAAGGGCCTT TCCGTCCTCA GCCGTCGCTT CATGTGACTC CACGGAGTAC CGGGCGCCGT
1419 CCAGGCACCT CGATTAGTTC TCGAGCTTTT GGAGTACGTC GTCTTTAGGT TGGGGGGAGG
1479 GGTTTTATGC GATGGAGTTT CCCCACACTG AGTGGGTGGA GACTGAAGTT AGGCCAGCTT
1539 GGCACTTGAT GTAATTCTCC TTGGAATTTG CCCTTTTTGA GTTTGGATCT TGGTTCATTC
3′ end of Intron 1
1599 TCAAGCCTCA GACAGTGGTT CAAAGTTTTT TTCTTCCATT TCAGGTGTCG TGA...
5′ end of Exon 2

pEF6/His A, B, and C Vectors

Map of pEF6/His

The figure below summarizes the features of the pEF6/His vectors. **The sequences for pEF6/His A, B, and C can be downloaded from** www.invitrogen.com or requested from Technical Support (see page 18).



Comments for pEF6/His C
5822 nucelotides

EF-1α promoter: bases 474-1651
T7 promoter/priming site: bases 1668-1687
ATG initiation codon: bases 1716-1718
Polyhistidine (6xHis) region: bases 1728-1745
Xpress™ epitope: bases 1785-1808
Enterokinase recognition site: bases 1794-1808
Multiple cloning site: bases 1808-1887
BGH reverse priming site: bases 1900-1917
BGH polyadenylation sequence: bases 1903-2130
f1 origin of replication: bases 2176-2604
SV40 promoter and origin: bases 2631-2939
EM-7 promoter: bases 2986-3041
Blasticidin resistance gene (ORF): bases 3060-3458
SV40 polyadenylation sequence: bases 3616-3746
pUC origin: bases 4130-4803
Ampicillin resistance gene (ORF): bases 4948-5808 (complementary)

* *Bsu*36 I is unique to version B.

** There is a stop codon following the *Xba* I site in version A.

† There is a stop codon in the *Pme* I site in version C.

Continued on next page

pEF6/His A, B, and C Vectors, Continued

Features of pEF6/His

pEF6/His A (5,823 bp), pEF6/His B (5,824 bp), and pEF6/His C (5,822 bp) contain the following elements. All features have been functionally tested.

Feature	Benefit
Human elongation factor 1α (hEF-1α) promoter	Permits overexpression of your recombinant protein in a broad range of mammalian cell types (Goldman *et al.*, 1996; Mizushima and Nagata, 1990).
T7 promoter/priming site	Allows for *in vitro* transcription in the sense orientation and sequencing through the insert.
N-terminal polyhistidine tag	Permits purification of your recombinant protein on metal-chelating resin such as ProBond™.
Xpress™ epitope tag	Allows for the detection of an 8 amino acid epitope (Asp-Leu-Tyr-Asp-Asp-Asp-Asp-Lys) on the recombinant protein with the Anti-Xpress™ Antibody.
Enterokinase cleavage site	Allows for the removal of the N-terminal polyhistidine tag from the recombinant protein using an enterokinase such as EKMax™ Enterokinase (see page 17).
Multiple cloning site in three reading frames	Allows insertion of your gene and facilitates cloning in-frame with the polyhistidine N-terminal tag and the Xpress™ epitope.
BGH reverse priming site	Permits sequencing through the insert.
Bovine growth hormone (BGH) polyadenylation signal	Efficient transcription termination and polyadenylation of mRNA (Goodwin and Rottman, 1992).
f1 origin	Allows for the rescue of single-stranded DNA.
SV40 early promoter and origin	Allows efficient, high-level expression of the blasticidin resistance gene and episomal replication in cells expressing SV40 large T antigen.
EM-7 promoter	Allows for the expression of the blasticidin resistance gene in *E. coli.*
Blasticidin resistance gene (*bsd*)	Selection of transformants in *E. coli* and stable transfectants in mammalian cells (Kimura *et al.*, 1994).
SV40 polyadenylation signal	Efficient transcription termination and polyadenylation of mRNA.
pUC origin	High-copy number replication and growth in *E. coli.*
Ampicillin resistance gene (β-lactamase)	Selection of transformants in *E. coli.*

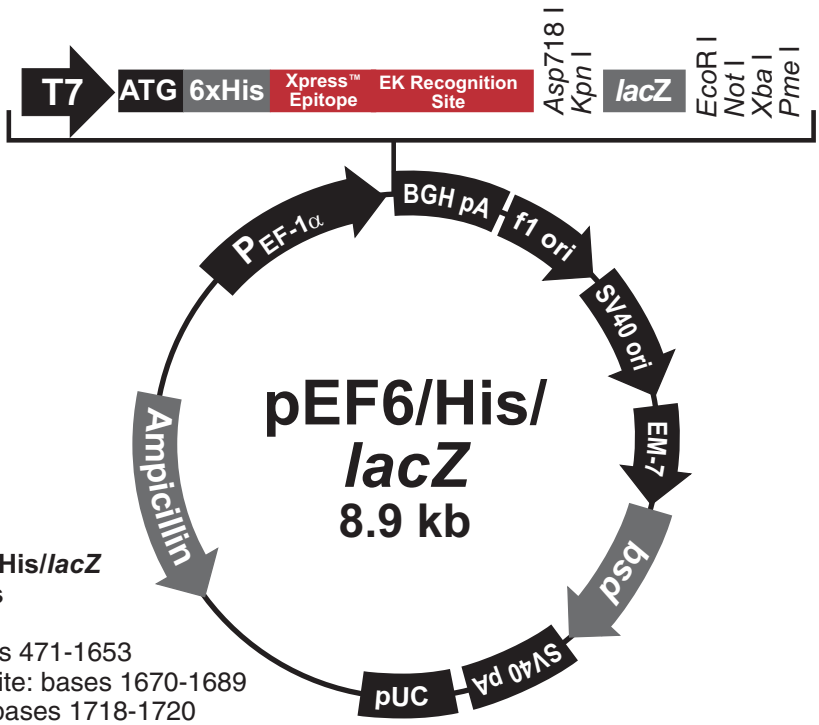
pEF6/His/*lacZ*

Description

pEF6/His/*lacZ* is a 8,889 bp control vector containing the gene for β-galactosidase. pcDNA6/His/*lacZ* was digested with *Sca* I and *Kpn* I to remove the CMV promoter. A 2.1 kb *Sca* I-*Kpn* I fragment containing the EF-1α promoter was ligated into pcDNA6/ His/*lacZ* to create pEF6/His/*lacZ*.

Map of Control Vector

The figure below summarizes the features of the pEF6/His/*lac*Z vector. **The sequence for pEF6/His/*lacZ* can be downloaded from www.invitrogen.com** or requested from Technical Support (see page 18).



Comments for pEF6/His/*lacZ*
8889 nucelotides

EF-1α promoter: bases 471-1653
T7 promoter/priming site: bases 1670-1689
ATG initiation codon: bases 1718-1720
Polyhistidine (6xHis) region: bases 1730-1747
Xpress™ epitope: bases 1787-1810
Enterokinase recognition site: bases 1796-1810
lacZ portion of fusion: bases 1839-4888
BGH reverse priming site: bases 4966-4983
BGH polyadenylation sequence: bases 4969-5196
f1 origin of replication: bases 5242-5670
SV40 promoter and origin: bases 5697-6006
EM-7 promoter: bases 6054-6109
Blasticidin resistance gene (ORF): bases 6128-6526
SV40 polyadenylation sequence: bases 6684-6814
pUC origin: bases 7197-7870
Ampicillin resistance gene (ORF): bases 8015-8875 (complementary)

Accessory Products

Additional Products

The following additional products may be used with the pEF6/His vectors. For more information, visit www.invitrogen.com or contact Technical Support (see page 18).

Item	Quantity	Cat. no.
Electrocomp™ Kit (TOP10F´)	2 × 20 reactions	C665-11
	6 × 20 reactions	C665-24
One Shot® TOP10F´ Chemically Competent *E. coli*	20 × 50 µL	C3030-03
One Shot® INVαF' Chemically Competent *E. coli*	20 × 50 µL	C2020-03
	40 × 50 µL	C2020-06
Ampicillin	200 mg	11593-027
Blasticidin	50 mg	R210-01
Carbenicillin	5 g	10177-012
T7 promoter primer	2 µg	N560-02
BGH Reverse primer	2 µg	N575-02
PureLink™ HiPure Plasmid Miniprep Kit	100 preps	K2100-03
PureLink™ HiPure Plasmid Midiprep Kit	25 preps	K2100-04
Lipofectamine™ 2000 Reagent	1.5 mL	11668-019
β–Gal Assay Kit	1 kit	K1455-01
β–Gal Staining Kit	1 kit	K1465-01
Anti-Xpress™ Antibody	50 µL	R910-25
EKMax™ Enterokinase	250 units	E180-01
ProBond™ Purification System	6 purifications	K850-01
ProBond™ Resin	50 mL	R801-01
	150 mL	R801-15

Restriction Enzymes

For your convenience, Invitrogen offers an extensive selection of restriction enzymes, including the following:

- *Ssp* I
- *Mlu* I
- *Sca* I

Visit www.invitrogen.com for more details.

Technical Support

Web Resources

Visit the Invitrogen website at **www.invitrogen.com** for:

- Technical resources, including manuals, vector maps and sequences, application notes, MSDSs, FAQs, formulations, citations, handbooks, etc.
- Complete technical support contact information
- Access to the Invitrogen Online Catalog
- Additional product information and special offers

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Continued on next page

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References

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1994). Current Protocols in Molecular Biology (New York: Greene Publishing Associates and Wiley-Interscience).

Chen, C., and Okayama, H. (1987). High-Efficiency Transformation of Mammalian Cells by Plasmid DNA. Molec. Cell. Biol. *7*, 2745-2752.

Chu, G., Hayakawa, H., and Berg, P. (1987). Electroporation for the Efficient Transfection of Mammalian Cells with DNA. Nucleic Acids Res. *15*, 1311-1326.

Felgner, P. L., Holm, M., and Chan, H. (1989). Cationic Liposome Mediated Transfection. Proc. West. Pharmacol. Soc. *32*, 115-121.

Felgner, P. L., and Ringold, G. M. (1989). Cationic Liposome-Mediated Transfection. Nature *337*, 387-388.

Goldman, L. A., Cutrone, E. C., Kotenko, S. V., Krause, C. D., and Langer, J. A. (1996). Modifications of Vectors pEF-BOS, pcDNA1, and pcDNA3 Result in Improved Convenience and Expression. BioTechniques *21*, 1013-1015.

Goodwin, E. C., and Rottman, F. M. (1992). The 3´-Flanking Sequence of the Bovine Growth Hormone Gene Contains Novel Elements Required for Efficient and Accurate Polyadenylation. J. Biol. Chem. *267*, 16330-16334.

Izumi, M., Miyazawa, H., Kamakura, T., Yamaguchi, I., Endo, T., and Hanaoka, F. (1991). Blasticidin S-Resistance Gene (*bsr*): A Novel Selectable Marker for Mammalian Cells. Exper. Cell Res. *197*, 229-233.

Kim, D. W., Uetsuki, T., Kaziro, Y., Yamaguchi, N., and Sugano, S. (1990). Use of the Human Elongation Factor 1 Promoter as a Versatile and Efficient Expression System. Gene *91*, 217-223.

Kimura, M., Takatsuki, A., and Yamaguchi, I. (1994). Blasticidin S Deaminase Gene from Aspergillus terreus (BSD): A New Drug Resistance Gene for Transfection of Mammalian Cells. Biochim. Biophys. ACTA 1219, 653-659.

Kozak, M. (1987) An Analysis of 5´-Noncoding Sequences from 699 Vertebrate Messenger RNAs. Nucleic Acids Res. 15, 8125-8148

Kozak, M. (1990) Downstream Secondary Structure Facilitates Recognition of Initiator Codons by Eukaryotic Ribosomes. Proc. Natl. Acad. Sci. USA 87, 8301-8305

Kozak, M. (1991) An Analysis of Vertebrate mRNA Sequences: Intimations of Translational Control. J. Cell Biology 115, 887-903

Miller, J. H. (1972). Experiments in Molecular Genetics (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory).

Mizushima, S., and Nagata, S. (1990). pEF-BOS, a Powerful Mammalian Expression Vector. Nucleic Acids Res. *18*, 5322.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, Second Edition (Plainview, New York: Cold Spring Harbor Laboratory Press).

Shigekawa, K., and Dower, W. J. (1988). Electroporation of Eukaryotes and Prokaryotes: A General Approach to the Introduction of Macromolecules into Cells. BioTechniques *6*, 742-751.

Takeuchi, S., Hirayama, K., Ueda, K., Sakai, H., and Yonehara, H. (1958). Blasticidin S, A New Antibiotic. The Journal of Antibiotics, Series A *11*, 1-5.

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References, Continued

Uetsuki, T., Naito, A., Nagata, S., and Kaziro, Y. (1989). Isolation and Characterization of the Human Chromosomal Gene for Polypeptide Chain Elongation Factor-1 . J. Biol. Chem. *264*, 5791-5798.

Wigler, M., Silverstein, S., Lee, L.-S., Pellicer, A., Cheng, Y.-C., and Axel, R. (1977). Transfer of Purified Herpes Virus Thymidine Kinase Gene to Cultured Mouse Cells. Cell *11*, 223-232.

Yamaguchi, H., Yamamoto, C., and Tanaka, N. (1965). Inhibition of Protein Synthesis by Blasticidin S. I. Studies with Cell-free Systems from Bacterial and Mammalian Cells. J. Biochem (Tokyo) *57*, 667-677.

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